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I, Norio Ohmori, registered patent attorney in Japan having the address at Fukuoka Building, 9th Floor, 8-7, Yaesu 2-chome, Chuo-ku, Tokyo 104-0028 Japan, am the translator of the documents attached and I state that the following is a true translation to the best of my knowledge and belief.

Signature of Translator

Norio Ohmori

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[Inventor]

[Domicile or Residence] 2-16-20, Suizenji, Kumamoto-shi, Kumamoto

[Name] YAMAMURA, Ken-ichi

[Inventor]

[Domicile or Residence] 55-2-1103, Yoyasu-machi, Kumamoto-shi, Kumamoto

[Name] ARAKI, Kimi

[Applicant]

[Identifying Number] 591023158

[Name] KUMAMOTO TECHNOPOLIS FOUNDATION

[Agent]

[Identifying Number] 100099508

[Patent Attorney]

[Name] KATO, Hisashi

[Telephone Number] 092-413-5378

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[Name of Document] DESCRIPTION

[Title of the Invention] TRAP VECTORS AND GENE TRAPPING METHOD USING THE SAME

[Scope of the Claim]

[Claim 1] A trap vector for use in gene trapping utilizing Cre-loxP, wherein a mutation is introduced into the left side repeat sequence of a loxP sequence of said trap vector so that no further recombination would occur with other loxP when said loxP sequence has once recombined with a lox having a mutation in its right side repeat sequence.

[Claim 2] The trap vector according to claim 1, wherein said loxP into which a mutation is introduced has the mutation of TACCGTTCGTATA (normally ATAACTTCGTATA) introduced into its left side repeat sequence.

[Claim 3] The trap vector according to claim 2, wherein said trap vector is composed of a spacer, a splice acceptor, lox71, IRES, beta-geo, loxP, pUC19 and a spacer positioned in this order from the 5' end, or a spacer, lox71, IRES, beta-geo, loxP, pUC19 and a spacer positioned in this order from the 5' end.

[Claim 4] A method of gene trapping, comprising using the trap vector according to any one of claims 1 to 3.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[TECHNICAL FIELD TO WHICH THE INVENTION PERTAINS]

The present invention relates to random mutation ES clone technology using gene trapping method.

[0002]

[BACKGROUND ART]

It is said that structural analysis of human genome will be completed in or before 2003 as the human genome project is progressing well. Now, the age of isolating genes one by one and analyzing their structures separately seems to be over, and we have come into the age of "functional analysis" of genome.

[0003]

With the nucleotide sequence of genome alone, however, information on functions is insufficient, thus, a novel analysis system for functional analysis is needed. Although one of the major goals of human genome analysis is to elucidate causative genes in human diseases, such diseases cannot be explained with the structures of causative genes alone. Further, it is impossible to carry out experiments by using human patients and analyze the mechanism of pathogenesis.

[0004]

Accordingly, production of model individuals is an indispensable assignment in order to analyze processes of disease development and to develop new treatment methods after the identification of causative genes.

[0005]

On the other hand, if genome is divided into gene regions and non-gene regions in terms of structure, it is considered that these two parts have separate functions and that it is necessary to analyze the functions of both parts (Fig. 1). From the viewpoint of entire genome, it is considered that each gene is performing only a part of the entire function and genome is not a mere collection of genes and may have unknown functions. In fact, a new concept, "position effect mutation" has been established. From this, it is presumed that genome has regions of unknown function.

[0006]

Gene regions also are composed of regulatory regions and coding regions and at present, the target of genome functional analysis is coding regions. Functional analysis of the regulatory region will be important in the future. Because when mouse is compared with human, the kinds of genes they have are almost equal. Despite it, there is difference in species between mouse and human. It is believed that this difference is not due to difference in protein but due to difference in the regulation of gene expression. A transcription factor or the like involved in the regulation of gene expression can be elucidated from the sequence of the coding region of the relevant gene. The analysis of those elements to which the transcription factor binds is extremely difficult at present because a number of those elements exist in the regulatory region of one gene. However,

as a technique of functional analysis, a method using bacterial artificial chromosomes or the like may be considered.

[0007]

It is considered that functional analysis of coding regions may be performed at the mRNA level, protein level, cell level, tissue/organ level and individual level. It is believed that such analysis at the mRNA level can be performed using DNA chips, and on the other hand, the use of ES cells seems to be the best way for performing functional analysis at other levels. Because various cell and tissue derivative systems have been developed directly from ES cells *in vitro* and a number of such systems are expected to be developed in the future. Furthermore, the use of ES cells is advantageous in that individual level analysis systems can be established.

[0008]

From the foregoing, it is understood that gene knockout at ES cell level and production of the said knockout mice are extremely important in functional analysis of genome. To date, homologous recombination method using ES cells has played a major role in the production of knockout mice. However, considering this method not as a strategy of producing knockout mice separately but from a strategic viewpoint of producing knockout mice comprehensively, this method has serious problems.

[0009]

First, this method requires too much time. The rate-determining step in the production of knockout mice is to isolate knockout ES clones generated through homologous recombination using ES cells. Even a skillful researcher needs at least three months for isolating a knockout ES clone. Thus, only four genes can be knocked out in one year. Accordingly, in the case of introducing each one mutation into 10^5 genes, 25,000x researchers are required for one year. It is estimated that approximately 1,000 lines of knockout mice are produced in one year in the world. Based on the above estimation, it is expected to produce them actively in 250 places. This means that it would take 100 years to produce 10^5 knockout ES clones. This is so unrealistic compared to the advance in the structural analysis of human genome that is to be completed in 2003.

[0010]

Secondly, this method requires too much cost. At least 2 to 4 million yen is necessary to produce one line of knockout mouse excluding personnel expenses and depreciation expenses. Thus, production of 10^5 simple knockout mice requires 200 to 400 billion yen.

[0011]

As described above, the conventional homologous recombination using ES cells has problems, and genome is vast. However, the number of genes in genome is limited. Therefore, knockout mice are directly connected with future development of epoch-making drugs and have extremely high added value. Under circumstances, it has become the world's "strategy" to produce mutant mice "at random" and "in large scale". At present, the three methods described below are considered most reasonable in the production of random mutation mice.

[0012]

The first one is a method using ethylnitrosourea (ENU) which is a mutagenic compound. When ENU is administered to adult male mice, ENU acts on spermatogoniums before meiosis and causes about 50 to 100 point mutations per spermatogonium at random. Therefore, by crossing one treated male mouse with one normal female mouse, many kinds of mutant mice can be produced in F1 generation. Mutations occur at a frequency of about 1/1,000/gamete per locus. In the method using ENU, if 1,000 mice are screened for a specific locus, one mouse has a mutation caused in that locus in terms of probability, thus, this method is considered highly efficient. In the case of this ENU, point mutations may be caused.

[0013]

The second method is a method using chlorambucil that is also a mutagenic compound. This method causes mutations in spermatogoniums at the same frequency as in the method using ENU. However, these mutations are deletion mutations, and sometimes as many as one megabases may be deleted.

[0014]

The third method is a method using gene trapping. It is a technique that was developed for the purpose of searching for unknown genes by introducing trap vectors containing a marker gene having no promoter into ES cells and then monitoring the expression of the marker gene as an index. Trap vectors are integrated into ES cells at random and, as a result of their integration, endogenous genes are disrupted in most cases. Therefore, preparing chimeric mice from such ES cells can produce various knockout mice.

[0015]

However, each of the methods using a mutagenic compound and the method using gene trapping has an advantage(s) and a drawback(s) (Fig.2).

According to the ENU method, production of mutant mice is easy, but establishment of individual mutant lines is not easy because segregation by crossing should be conducted. Further, in order to identify mutated genes, the relevant locus should be identified first by linkage analysis using polymorphic DNA markers, and then the gene should be isolated by positional cloning method.

[0016]

According to the chlorambucil method, production of mutant mice is also easy, but deleted sites should be identified. For that purpose, analysis must be made using a number of polymorphic DNA markers. Besides, generally, methods using a mutagen need large breeding rooms. Although the gene trapping method requires labor and technology for producing mutant mice, identification of mutated genes is easy and experiments can be conducted according to the size of breeding rooms. Gene trapping ES clones *per se* are precious resource for functional analysis of genome. The gene trapping method is also remarkably different from other methods in this point.

[0017]

A project of large-scale mutant production using ENU has been started in Europe. In Germany, Dr. Balling of the Institute of Mammalian Genetics and others started this project in 1997 as a part of the human genome project. In England, supported by SmithKline Beecham, Dr. Brown and others started this project at MRC Mouse Genome Center in Harwell aiming at establishment of mutant mice having mutations mainly in brain/nervous

system. To date, these two groups have established approximately 200 lines of mutant mice exhibiting dominant inheritance. The project is proceeding more efficiently than expected. In the United States, it has been decided that structural analysis of mouse genome and production of mutant by the ENU method start with a huge budget (6 billion yen/year) at Case Western Reserve University, Oak Ridge National Laboratory, etc.

[0018]

Some laboratories in the world have already started production of mutants by gene trapping. In the United States, a private firm Lexicon Genetics Incorporated is undertaking random disruption by gene trapping using retrovirus vectors. However, ordinary researchers can hardly use this service because of the following reasons. Briefly, it is not sure whether an endogenous gene is disrupted or not even if the gene is trapped; it is not clear whether germline chimeric mice can be produced; an additional charge is required for the production of chimeric mice; and considerable charges are required for using the service. In Germany, gene trapping is performed toward a goal of 12,000 clones as a part of the ENU project. Anyway, these are proceeding focusing on the analysis of trapped genes rather than the establishment of mouse lines.

[0019]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

The problem to be solved by the invention is to overcome the problems that conventional gene trapping methods have, to develop a novel "exchangeable gene trapping method" that seems almost ideal, to establish ES trap clones in large scale using the above method, and to produce mouse mutants using the trap clones.

[0020]

[MEANS FOR SOLVING THE PROBLEMS]

As a result of intensive and extensive researches toward the solution of the above problems, the present inventors have reached an idea of using the bacteriophage-derived recombination system Cre-loxP in gene trapping method and thus, the present invention has been achieved. Cre is a recombinase that recognizes a loxP sequence and causes recombination at that site.

[0021]

Conventional gene trapping method is a method utilizing the fact that trap vectors introduced into ES cells are integrated into mouse endogenous genes incidentally and at random. Trap vectors are generally composed of a reporter gene with a splice acceptor alone, a drug selection marker gene and a plasmid. Only when these vectors are integrated downstream of a mouse endogenous gene, the reporter gene is expressed. This means that it is possible to know the vector's integration into an endogenous gene by monitoring the expression of the reporter gene in the trap vector. By utilizing a plasmid of the trap vector, the trapped endogenous gene can be isolated and since the endogenous gene is disrupted at the time of trapping, knockout mice can be produced immediately. Further, since the reporter gene is expressed under the control of the expression regulatory region of the mouse endogenous gene, the tissue specificity and time specificity of the expression of the endogenous gene can be analyzed easily. However, even if a mouse endogenous gene could be disrupted completely, it has been impossible to introduce thereinto subtle mutations, such as single amino acid substitution, seen in human hereditary diseases. Also, it has been impossible to replace the disrupted mouse gene with a human gene.

[0022]

Toward the solution of these problems, the present invention has modified the Cre-loxP system, which is a bacteriophage-derived recombination system and utilized it in the trap vector in gene trapping. As a result, it has become possible to insert any gene into a mutant loxP site of the trap vector after a mouse gene has been disrupted as a result of the integration of the trap vector. According to the present invention, it has become possible to introduce subtle mutations, such as single amino acid substitution, seen in human hereditary diseases. It has also become possible to replace the trapped gene with a human gene.

[0023]

loxP consists of a 34 bp sequence and has 13 bases of inverted repeat sequences at both side and 8 bp spacer located in the center of the sequence. In bacteria, recombination occurs between two loxP sites, and insertion or deletion reaction takes place. If it is possible to cause insertion reaction in mammal cells, then any desired gene can be inserted

later. This would dramatically expand the applicability of gene trapping. Actually, since mammal cells have large nuclei, circular DNA molecules with once deleted loxP will diffuse and insertion reaction is hardly observed.

[0024]

In order to cause insertion reaction, the present inventors have elaborated a method in which mutations are introduced into the loxP sequence and, once a gene has been inserted into genome, the gene does not undergo deletion. For this method, the inventors have prepared two mutant loxP sequences.

[0025]

Briefly, the inventors created one mutant by introducing mutations, TACCGTTCGTATA (underlined portion was changed, the original sequence is ATAACTTCGTATA) into the left side of a repeat sequences of loxP and the mutant was designated "lox71". The other mutant was created by introducing mutations, TATACGAACGGTA (underlined portion was changed, the original sequence is TATACGAAGTTAT) into the right side of the repeat sequence of loxP and the mutant was designated "lox66". When recombination has occurred between lox71 on genome and lox66 on a plasmid, lox71/66 (TACCGTTCGTATA GCATACAT TATACGAACGGTA) is located at the left side of the repeat sequence and wild-type loxP (ATAACTTCGTATA GCATACAT TATACGAAGTTAT) is located at the right side of the repeat sequence. As a result, Cre no longer can recognize lox71/66, thus cannot cause recombination with loxP and remains inserting (Fig. 3).

[0026]

Actually, when lox71 has been integrated into ES cells in advance, and a plasmid containing lox66 is introduced thereinto, it revealed that the plasmid is integrated into the genome. Therefore, if this lox71, for example, has been integrated into a gene trapping vector in advance, it becomes possible to insert any desired gene later by using lox66. Thus, according to the present invention, it has become possible to replace the trapped gene with a gene into which a subtle mutation(s) has (have) been introduced or a human gene.

[0027]

Gene trapping vector U8 using this lox71 was constructed (see Fig. 4 and 5). The basic part of U8 is derived from pGT1.8IRESbetageo. This pGT1.8IRESbetageo contains mouse En-2 gene-derived splice acceptor, IRES and beta-geo. lox71 is inserted into the BglII site of this pGT1.8IRESbetageo followed by SalI treatment to thereby provide a SalI fragment. On the other hand, plasmid pEBN-Seti is prepared by inserting a 180 bp spacer sequence (which is derived from rabbit beta-globin gene and may be used to protect both ends from deletion of important regions when trapp vector is inserted into mouse genome, and which may have any sequence), loxP and poly(A) signal into pUC19.

[0028]

The SalI fragment obtained above was inserted into the SalI site of this plasmid to produce U8. Thus, the structure of this trapping vector is expressed spacer, splice acceptor, lox71, IRES, beta-geo, loxP, pUC19 and spacer from the 5' end in this order. Also, U8delta whish is deleted the splice acceptor from U8 was obtained by the same protocol. This vector has a structure in which lox71 which has been mutated at the left side of the repeat sequence of loxP is linked before the reporter beta-geo and loxP after beta-geo. This is because the intermediate IRES and beta-geo portion can be removed completely by transiently expressing Cre after the vector has been integrated. As a result, plasmid pUC19 is located close to the mouse endogenous gene which was located upstream of the plasmid. Thus, the mouse endogenous gene can be isolated easily.

[0029]

The conventional gene trapping at the first step is performed using this vector. By these procedures, the endogenous gene in the ES cells is disrupted. Using these ES cells, the knockout mice can be prepared. After isolation of the trapped endogenous gene, subtle mutations are introduced into this gene in *E. coli* using site-specific mutagenesis or the like and the gene is ligated downstream of lox66 on the plasmid. By introducing it into ES cells, this modified gene can be introduced into lox71 in the second step (Fig.6). According to these procedures, not only modified endogenous genes but also human genes may be introduced. Any gene may be introduced and in the present invention, this method was designated exchangeable gene trapping method.

[0030]

(Screening system)

If a gene trapping vector was introduced into ES cells and then neomycin resistant clones have been selected, these clones are considered to have the trap vector integrated downstream of a mouse endogenous gene. DNA is extracted from these clones and analyzed by Southern blotting, to thereby select clones in which only one copy of the trap vector is integrated. The inventors have found that this selection method enables efficient selection of mouse gene-trapping clones. Therefore, this will be used as a screening system in the present invention.

[0031]

(The procedure for preparing a gene trapping mutant (Fig. 7))

1) Isolation of Neomycin Resistant Clones

One hundred μg of trap vector is introduced into $3x10^7$ TT2 ES cells suspended in 0.8 ml of phosphate buffer by electroporation method (using a BioRad GenePulser at 800 V and 3 microF), and the resultant cells are cultured in the presence of G418 which concentration is 200 microgram /ml. After 1 week, neomycin resistant clones are isolated.

2) Selection of ES Clones by Integration Pattern

DNA is extracted from ES clones by conventional methods, and integration patterns are analyzed by Southern blotting. The vector which is integrated only one copy is selected. These procedures are performed in order to select those clones in which isolation of mouse endogenous genes by plasmid rescue will be easy. Also, those clones which have become neomycin resistant with only one copy of the vector are trapping mouse endogenous genes at an extremely high probability.

3) Establishment of Trap Lines by Production Of Chimeric Mouse

Selected ES cells are aggregated with ICR- derived morulae to prepare chimeric mice embryos. The resultant embryos are transferred into the uterus of a foster female mouse that has been brought into a pseudo-pregnant state by mating with a sterile male mouse.

Those are produced by standard methods. Offspring will be born about 17 days after this transfer and chimeric mice are selected, such chimeric mice is crossed with normal female mice to obtain F1 to thereby establish mutant animal lines. The following analysis is conducted only for those mice that have been established as trap lines.

4) Freezing Spermatozoa and Two-cell Stage Embryos

In vitro fertilization is carried out using spermatozoa from F1 and it and when two-cell stage embryos obtained, they can be stored frozen by ultra-quick freezing technique.

5) Analysis of Expression Patterns

F1 mice are crossed and then expression patterns in 9.5-day embryos and adult mice are analyzed.

6) Analysis of Phenotypes

For each of the established mouse lines, phenotypes of heterozygous and homozygous mice are analyzed. This analysis of phenotypes is carried out by macroscopic observation, internal observation by anatomy, microscopic examination of tissue sections from each organs, examination of the skeletal system by X-ray photography, examination of behavior and memory, and blood examination.

7) Isolation and Structural Analysis of the Trapped Gene and Preparation of Chromosome Map

Isolation and determination of the nucleotide sequence of mouse DNA is carried out by using the trap clone and homology search is performed. With respect to the result, the sequence of the trapped DNA is classified into one of the groups of known genes, EST, unknown genes or repeats. As to the EST or unknown gene, a chromosome map can be prepared. Chromosome maps may be prepared by fluorescent *in situ* hybridization (FISH) or association analysis using microsatelite probes or the like. Once the position of the DNA on the chromosome has been determined, this position is compared with the positions of mutant genes in existing mutant mice to examine if the relevant position coincides with one of them.

8) Construction of Database

For each of the established lines, database is prepared on expression patterns of the

marker gene in 10-day embryos and adult animals; phenotypes in F1 and F2 mice; the nucleotide sequence of the trapped endogenous mouse DNA and, if the DNA is an EST or unknown gene, its position in the chromosome.

[0032]

(The Result of Pilot Studies)

(1) Overall Efficiency

1) Screening by Formation of Embryoid Bodies

One hundred and six neomycin resistant clones were suspension-cultured for the formation of embryoid bodies. The expression of b-gal was analyzed at the two point which are the stage of ES cells and after the induction of differentiation. As a result, it was found that 90 trap clones (86%) were expressing b-gal at any one of the above stages.

2) Selection of Clones Indicating Single Copy Integration

DNA was extracted from 109 trap clones that had expressed the marker gene during the process of embryoid body formation, and then integration patterns of the trap vector were analyzed. As a result, 76 clones (70%) had a single copy integrated. Of these, 27 clones (25%) were complete and 38 clones (35%) lacked pUC. Even if pUC was lost, it could be re-inserted by using lox71 site. Therefore, these 65 clones (60%) were found to be useful.

3) Efficiency of Germline Chimera Production

Chimeric mice were produced using the above-mentioned trap clones. As a result, germline chimeric mice were obtained from approximately one half of the clones.

4) Summary of the Entire Experiment

It was found that about 26% of the neomycin resistant clones selected initially reached the final stage of the experiment. Since the efficiency of germline chimera production is now increasing, it is believed that the overall efficiency can be increased further. However, the efficiency achieved at this time seems to be sufficient for the practice of researches.

[0033]

(2) Efficiency of the Gene Trapping Method

As a result of the pilot studies, 24 trap lined were established. Of these, 13 lines have proceeded to analysis at the gene level. Nucleotide sequences of these lines were

compared with GenBank and EMBL databases using BLAST program. The results were as follows: 9 clones were known genes; 3 clones were ESTs; and the remaining 1 clone was an unknown gene. According to the reports so far made by other researchers, 10-25% of trapped genes are known genes; 10-20% are ESTs; 50-80% are unknown genes; and 2-10% are repeats.

[0034]

(3) Trapped Genes

The inventors examined whether those genes involved in development and cell growth had been efficiently trapped or not by ascertaining the kinds of known genes by a screening method utilizing formation of embryoid bodies. As a result, it was found that the known genes were CBP (CREB binding protein) and Sp1 that are transcription factors; cyclin B2 involved in cell cycle; Crk and pHPS1-2 involved in signal transduction; rRNA, suil, hnRNP L and RNA polymerase I involved in translation; and mitochondrial DNA (Fig. 8). Thus, it was found that very common genes were trapped. A major part of these genes are involved in cell growth and this suggests that the screening system utilizing formation of embryoid bodies works well.

[0035]

(4) Confirmation of Gene Disruption by Trapping

It is one of the major points whether endogenous genes have been actually disrupted or not by gene trapping. Thus, the inventors have analyzed the structure of the trap site for 6 known genes. As a result, it was found that the trap vector was inserted into the promoter region in one gene; into an exon in one gene; and into an intron in 4 genes. In all of them, the gene was completely or partially disrupted. Therefore, it has become clear that endogenous genes can be disrupted efficiently by the method of gene trapping of the invention (Fig. 9).

[0036]

[Effect of the Invention]

(1) Knockout mice can be produced efficiently.

The gene trapping vector is integrated into the ES cell genome at random.

Therefore, mere introduction of the trap vector into ES cells does not necessarily mean integration into a gene and the vector may be integrated into a non-gene region. However, since the trap vector contains a neomycin resistance gene which is a drug resistance gene, those cells in which it is expressed are neomycin (also called G418) resistant. In other words, those cells that survive in the presence of neomycin are expressing neomycin resistance gene. The neomycin resistance gene in the trap vector is expressed only when integrated downstream of a mouse gene which is being expressed in the ES cells. Thus, the expression of this neomycin resistance gene means that it has been integrated downstream of a mouse gene. In most cases, mouse genes are knocked out by the integration of the trap vector into mouse genes. Therefore, a mouse gene-knocked out mice can be produced by using the ES cells. That is, knockout mice can be produced efficiently by selection of neomycin resistant clones and selection of those clones in which a single copy of the trap vector is integrated. According to conventional homologous recombination methods, one researcher can produce only 4 lines of knockout mice in one year at his/her best. According to the method of the present invention, however, one researcher can establish as many as 240 lines in one year if, for example, he/she establishes 6 lines per week and works 40 weeks a year. Thus, the method of the invention is 60 times more efficient than conventional methods.

[0037]

(2) The method of the invention allows detailed analysis of gene functions.

In the method of exchangeable gene trapping of the present invention, it is possible to introduce any mutations later. Thus, it is possible to introduce mutations in advance into each part of a gene that seems to have a certain function. Then, the mutant gene-introduced mouse can be created followed by analysis of the phenotype. As a result, the function of each part of the genes can be easily analyzed.

[0038]

(3) The disease model mice which are closer to human can be produced.

As described above, it is possible to create disease model mice which are closer to human because a human gene having the same mutation as found in a human disease can be

introduced into mice replacing the mouse gene.

[0039]

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[BRIEF DESCRIPTION OF THE DRAWINGS]

[Fig. 1] It is a diagram showing the genome functional analysis.

[Fig. 2] It is a diagram showing the comparison between a method of mutagenic compound and a method of gene trapping.

[Fig 3] It is a diagram showing insertion of DNA fragment by mutant loxP.

[Fig.4] It is a diagram showing the construction of a trap vector pU-Hachi.

[Fig.5] It is a flow chart showing procedure for preparing the U8 trap vector.

[Fig.6] It is a diagram showing a method of exchangeable gene trapping.

[Fig.7] It is a flow chart showing the production of trapping mutant.

[Fig.8] It is a diagram showing the trapped gene.

[Fig.9] It is a diagram showing various positions of integration of trap vectors.

[Name of Document] ABSTRACT

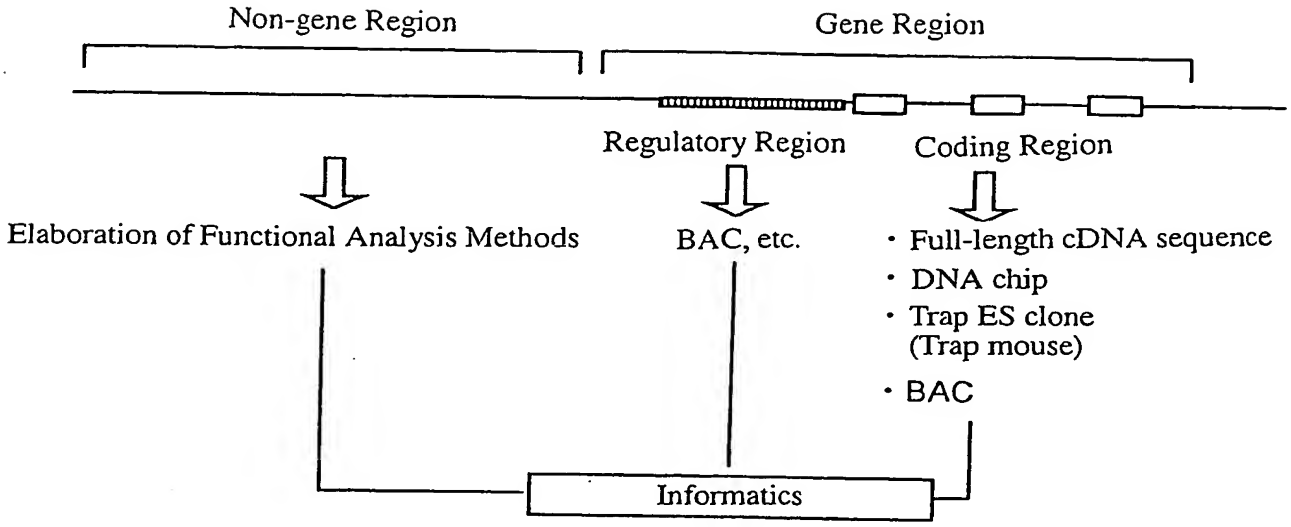
[ABSTRACT]

[PROBLEM] A novel exchangeable gene trapping method is developed and by using this method, the establishment of large scale ES trap clone and preparation of mouse mutants are carried out.

[MEANS FOR SOLUTION] A gene trapping is carried out by using a trap vector, wherein a mutation is introduced into the left side repeat sequence of a loxP sequence of said trap vector so that no further recombination would occur with other loxP when said loxP sequence has once recombined with a lox having a mutation in its right side repeat sequence.

[SELECTED DRAWING] FIG.6

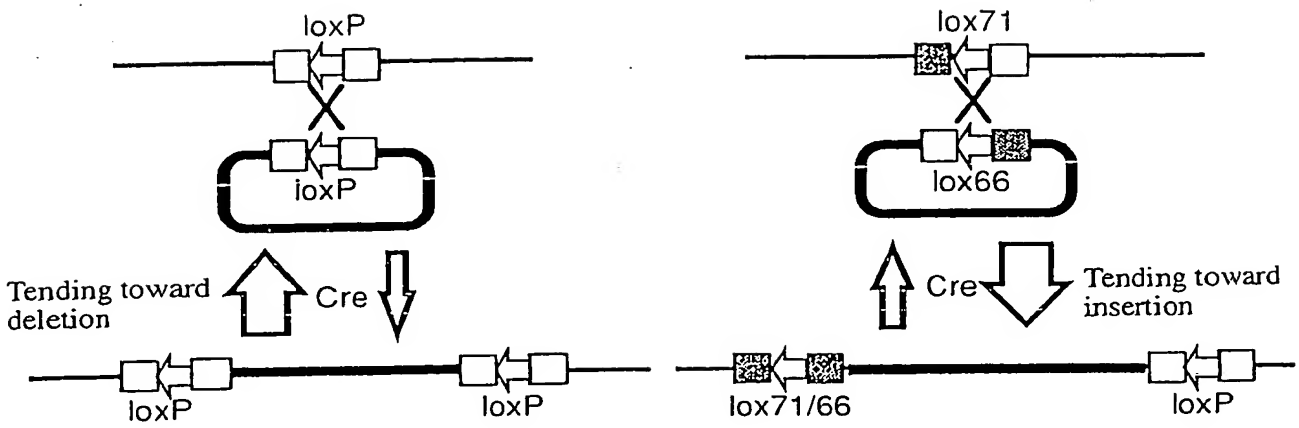
[Fig. 1]



[Fig.2]

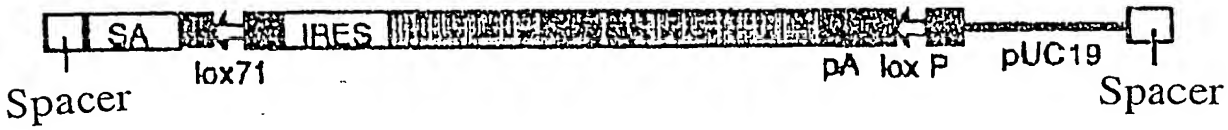
	ENU Method	Chlorambucil Method	Gene Trapping Method
Nature of Mutation	Point mutation	Deletion mutation	Any desired mutation
Production of Mutant Mouse	Easy	Easy	Difficult
Identification of Mutant Gene	Difficult	Medium	Easy
Other Features			Can use ES trap clones

[Fig. 3]



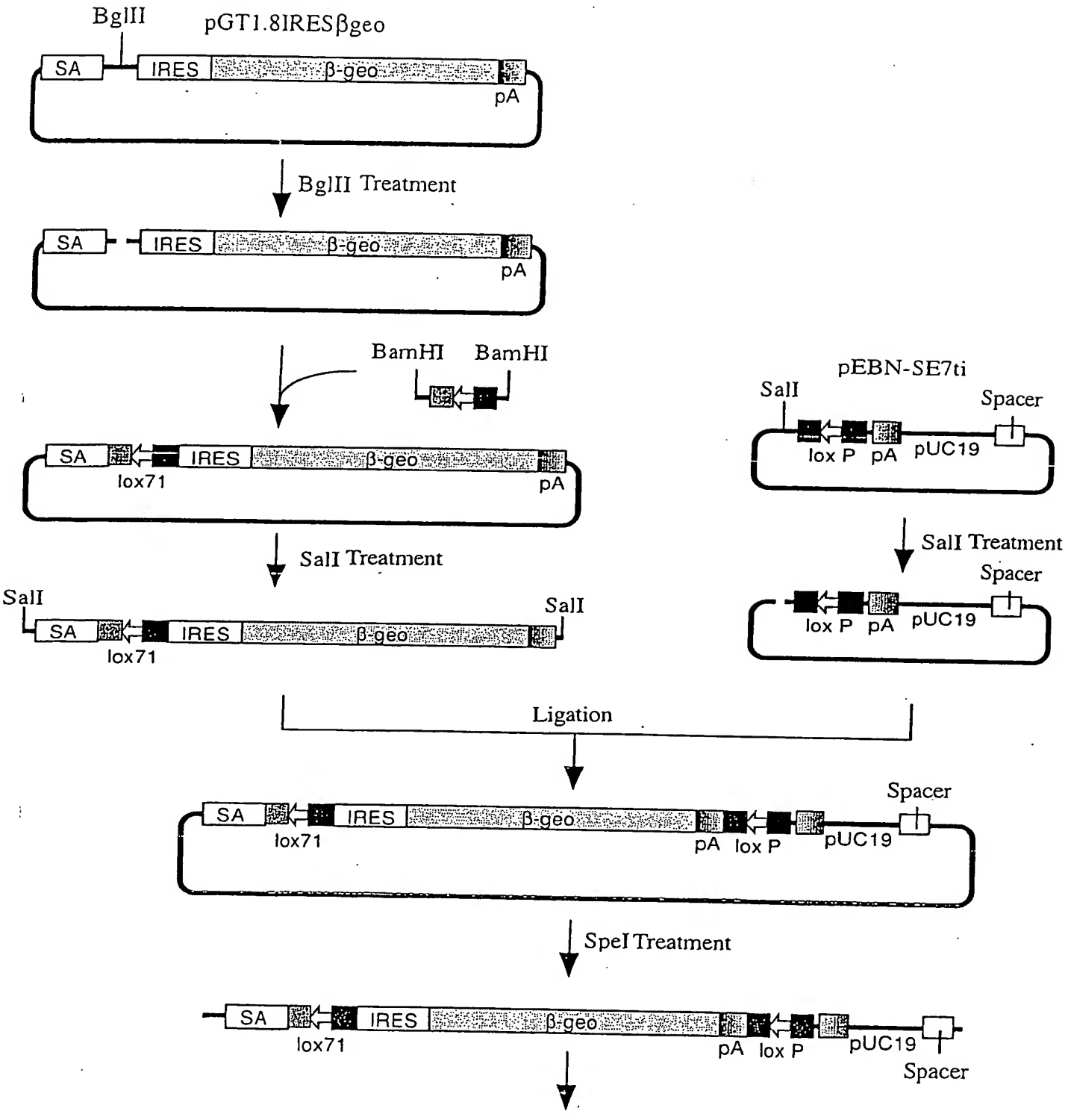
The left diagram shows normal reaction. The right diagram shows a recombinant reaction with mutant loxP. In the left diagram, because a circular DNA including an excised loxP is physically left, the reaction tends toward deletion rather than insertion. On the other hand, when the lox71 is used in the chromosome and the lox66 in circular DNA, a Cre recombinase hardly recognize a lox71/66 generated by recombination, thus the reaction tends towards insertion rather than deletion.

[Fig. 4]

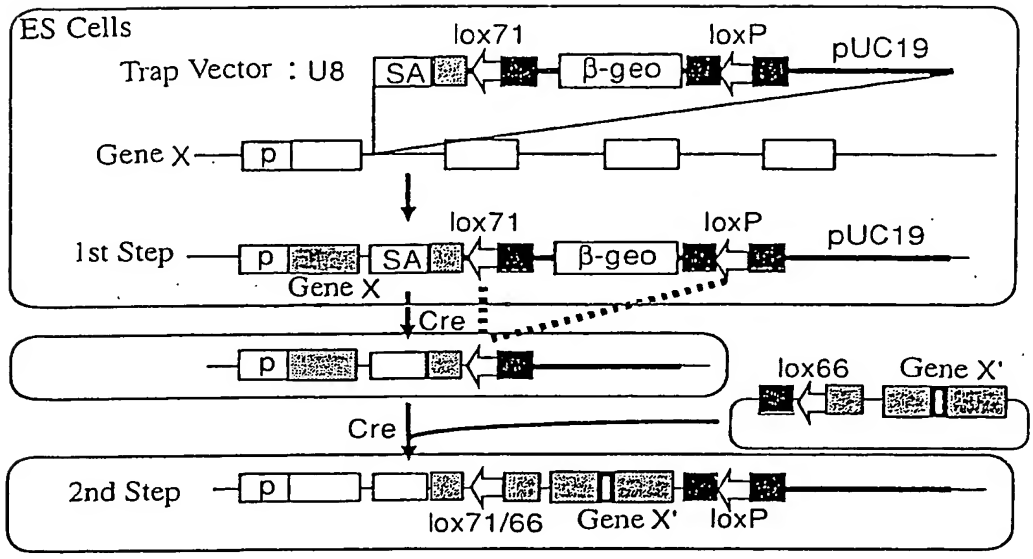


A spacer sequence may be any sequence to prevent insertion of deleted splice acceptor (SA). When the IRES (internal ribosomal entry site) is present, the translation will start from the site and β-geo protein will be produced.

[Fig. 5]

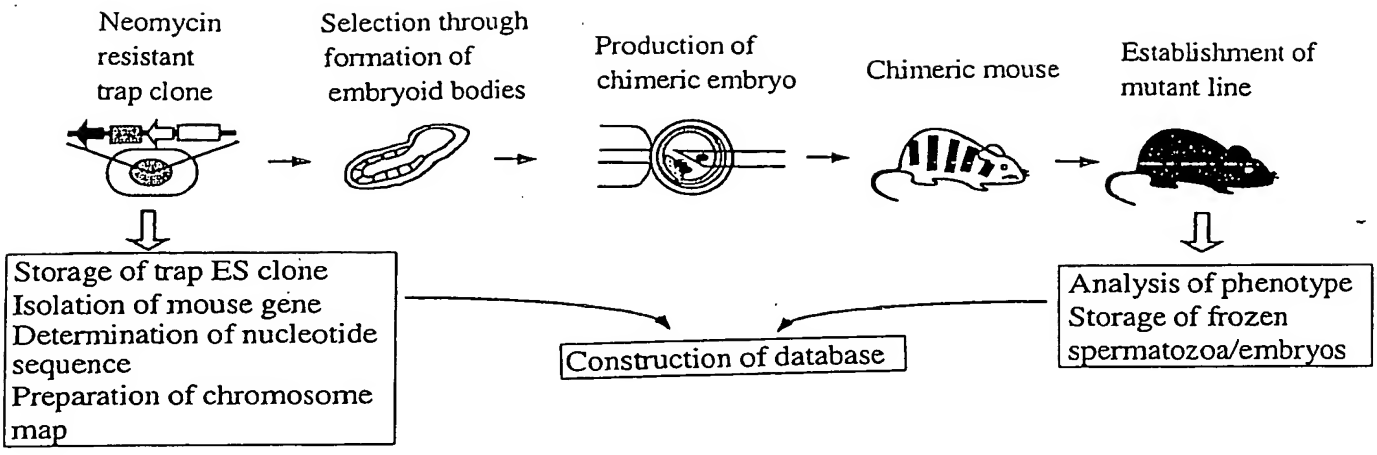

BglII
pGT1.8IRESβgeo
SA
IRES
β-geo
pA
BglII Treatment
BamHI
BamHI
lox71
SalI
SalI Treatment
pEBN-SE7ti
Spacer
lox P
pUC19
Ligation
SpeI Treatment
Introduction into ES Cells

[Fig. 6]



The conventional first-step gene trapping is carried out by using U8 trap vector. The gene knockout mice are prepared by the ES. After isolation of the endogenous gene, subtle mutations are introduced into the gene followed by integration downstream of lox66. In the second step, the gene can be inserted into lox71.

[Fig. 7]

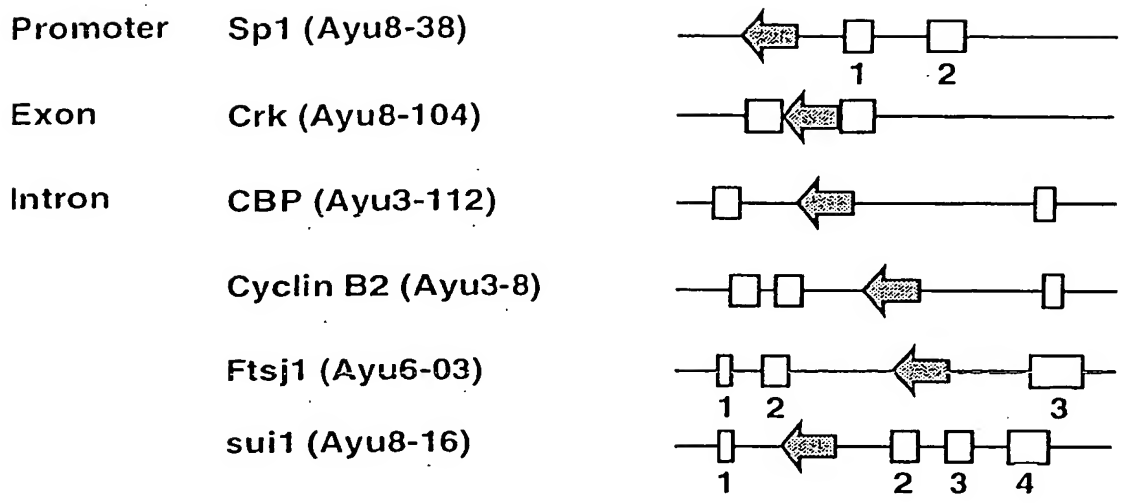

Neomycin resistant trap clone
Selection through formation of embryoid bodies
Production of chimeric embryo
Chimeric mouse
Establishment of mutant line
Storage of trap ES clone
Isolation of mouse gene
Determination of nucleotide sequence
Preparation of chromosome map
Construction of database
Analysis of phenotype
Storage of frozen spermatozoa/embryos

[Fig. 8]

Table 3

Class	Clone No.	Gene
1. Nucleus		
(1) Transcription	Ayu3-112	CBP
	Ayu8-038	Sp1
(2) Cell Cycle	Ayu3-008	Cyclin B2
	Ayu6-003	Homologous to the *E. coli* cell division protein Ftsj1
(3) Signal Transduction	Ayu8-104	Crk
	Ayu8-025	$pHPS_1$-2
(4) Cell Skeleton	Ayu8-003	dynamin II
2. Cytoplasm		
(1) Translation	Ayu3-022	rRNA
	Ayu8-016	sul1
	Ayu8-016	Upstream region of hnRNP L
	Ayu8-019	Very likely to be RNA polymerase I
(2) Others	Ayu3-001	Mitochondrial DNA
3. Unknown	Ayu7-003	Unknown

[Fig. 9]


Promoter
Exon
Intron
Sp1 (Ayu8-38)
Crk (Ayu8-104)
CBP (Ayu3-112)
Cyclin B2 (Ayu3-8)
Ftsj1 (Ayu6-03)
sui1 (Ayu8-16)
1
2
1
2
3
1
2
3
4